OFFICE OF NAVAL RESEARCH

CONTRACT N00014-88-C-0118

TECHNICAL REPORT 90-06

ATTENUATION OF IL-2 INDUCED MULTI-SYSTEM ORGAN EDEMA BY PHALLOIDIN AND ANTAMANIDE

BY

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25 JULY 1990

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER NBRL, BUSM 90-06	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) ATTENUATION OF IL-2 INDUCED MULTI-SYSTEM ORGAN EDEMA BY PHALLOIDIN AND ANTAMANIDE		5. TYPE OF REPORT & PERIOD COVERED Technical Report 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Richard Welbourn, Gideon Goldman, Lester Kobzik, C. Robert Valeri, Herbert B. Hechtman, and David Shepro		8. CONTRACT OR GRANT NUMBER(s) N00014-88-C-0118
9. PERFORMING ORGANIZATION NAME AND ADDRESS Naval Blood Research Laboratory Boston University School of Medicine 615 Albany St., Boston, MA 02118		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS Naval Medical Research and Development Command Bethesda, MD 20814		12. REPORT DATE 25 July 1990 13. NUMBER OF PAGES 21
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) Bureau of Medicine and Surgery Department of the Navy Washington, D.C. 20372		15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release and sale. Distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

No. 9: Departments of Surgery and Pathology, Brigham and Women's Hospital, Harvard Medical School, and The Biological Science Center, Boston University

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Attenuation of edema
Phalloidin
Antamanide
Interleukin (IL)-2
Cytokine

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Interleukin(IL)-2 is a potent cytokine with diverse effects including the ability to stimulate lymphocyte differentiation into cells capable of lysing tumor. Its therapeutic efficacy is limited because of side effects characterized by breakdown of the microvascular barrier, edema and weight gain, the mechanisms of which are largely unknown. Control of the microvascular barrier, thought to be a major function of the endothelial cell (EC), is in part regulated by cytoskeletal

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S/N 0102-LF-014-6601

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contractile proteins associated with the plasma membrane. This study tests whether the cyclopeptide phalloidin, which maintains actin filament organization and distribution and reduces macromolecular flux across the EC junction in vitro, would similarly maintain barrier tightness and prevent early edema produced by IL-2 in vivo. Anesthetized rats were treated at 30 min periods with IV:saline (0.5 ml,n=41); phalloidin (20 ug in 0.5 ml, n = 21); or antamanide, an analogue of phalloidin (20 ug in 0.5 ml, n = 21), starting 30 min prior to the 1 h infusion of 10^6U recombinant human IL-2 or saline. Six hours after the start of IL-2, there was edema in the saline/IL-2 group, as measured by increased wet to dry (W/d) ratios: in the lungs (W/d ratio 4.75 $\pm$0.14 relative to 3.94$\pm$0.20 in saline controls), heart (4.12$\pm$ 0.09 vs. 3.80$\pm$0.06) and kidney (4.36$\pm$0.03 vs. 4.18$\pm$0.05) (all $p < 0.05$). With saline/IL-2, bronchoalveolar lavage (BAL) fluid contained an elevated protein concentration of 1770$\pm$260 ug/ml, higher than 458$\pm$84 ug/ml in saline controls ($p < 0.05$, and plasma thromboxane (Tx)B_2 levels were raised to 1032$\pm$204 pg/ml, higher than 238$\pm$23 in saline controls ($p < 0.05$). Neutrophils were sequestered in the lungs 49$\pm$6 PMN/10 high power fields (HPF) relative to saline controls 21$\pm$2 PMN/10 HPF ($p < 0.05$). Phalloidin attenuated edema in lung (W/d ratio 4.47$\pm$0.07) and heart (3.82$\pm$0.07) (both $p < 0.05$) and reduced BAL protein leak to 955$\pm$20 ug/ml (n=6, $p < 0.05$). Antamanide treatment was as effective in limiting lung (W/d ratio 4.39$\pm$0.12) and heart edema (3.87$\pm$0.04) (both $p < 0.05$). In contrast to phalloidin, antamanide prevented kidney edema (4.14$\pm$0.09) (all $p < 0.05$) and did not lead to an alteration in the liver W/d ratio. Antamanide also prevented BAL fluid protein leak (482$\pm$13 ug/ml, $p < 0.05$). Both agents lowered plasma TxB2 ($p < 0.05$) but did not significantly affect lung sequestration of neutrophils. These data suggest that endothelial cytoskeleton may modulate early permeability induced by IL-2.

ABSTRACT

Interleukin (IL)-2 is a potent cytokine with diverse effects including the ability to stimulate lymphocyte differentiation into cells capable of lysing tumor. Its therapeutic efficacy is limited because of side effects characterized by breakdown of the microvascular barrier, edema and weight gain, the mechanisms of which are largely unknown. Control of the microvascular barrier, thought to be a major function of the endothelial cell (EC), is in part regulated by cytoskeletal contractile proteins associated with the plasma membrane. This study tests whether the cyclopeptide phalloidin, which maintains actin filament organization and distribution and reduces macromolecular flux across the EC junction in vitro, would similarly maintain barrier tightness and prevent early edema produced by IL-2 in vivo. Anesthetized rats were treated at 30 min periods with IV: saline (0.5 ml, n=41); phalloidin (20 μg in 0.5 ml, n=21); or antamanide, an analogue of phalloidin (20 μg in 0.5 ml, n=21), starting 30 min prior to the 1 h infusion of 10^6U recombinant human IL-2 or saline. Six hours after the start of IL-2, there was edema in the saline/IL-2 group, as measured by increased wet to dry (W/d) ratios: in the lungs (W/d ratio 4.75 ± 0.14 relative to 3.94 ± 0.20 in saline controls), heart (4.12 ± 0.09 vs. 3.80 ± 0.06) and kidney (4.36 ± 0.03 vs. 4.18 ± 0.05) (all $p < 0.05$). With saline/IL-2, bronchoalveolar lavage (BAL) fluid contained an elevated protein concentration of 1770 ± 260 μg/ml, higher than 458 ± 84 μg/ml in saline controls ($p < 0.05$), and plasma thromboxane $(Tx)B_2$ levels were raised to 1032 ± 204 pg/ml, higher than 238 ± 23 in saline controls ($p < 0.05$). Neutrophils were sequestered in the lungs 49 ± 6 PMN/10 high power fields (HPF) relative to saline controls 21 ± 2 PMN/10 HPF (p

<0.05). Phalloidin attenuated edema in lung (W/d ratio 4.47 ± 0.07) and heart (3.82 ± 0.07) (both $p < 0.05$) and reduced BAL protein leak to 955 ± 20 μg/ml ($n=6$, $p < 0.05$). Antamanide treatment was as effective in limiting lung (W/d ratio 4.39 ± 0.12) and heart edema (3.87 ± 0.04) (both $p < 0.05$). In contrast to phalloidin, antamanide prevented kidney edema (4.14 ± 0.09) (all $p < 0.05$) and did not lead to an alteration in the liver W/d ratio. Antamanide also prevented BAL fluid protein leak (482 ± 13 μg/ml, $p < 0.05$). Both agents lowered plasma TxB_2 ($p < 0.05$) but did not significantly affect lung sequestration of neutrophils. These data suggest that the endothelial cytoskeleton may modulate early permeability induced by IL-2.

INTRODUCTION

The efficacy of interleukin (IL)-2 immunotherapy has been established in various tumor models (8,17,18,22). However, significant toxicity, termed the vascular leak syndrome, limits IL-2 dosage and effectiveness in vivo. This syndrome is characterized by increased microvascular permeability and generalized edema (15). The time course of edema formation following IL-2 has been studied in several tumor models. In clinical trials of IL-2 used alone or in conjunction with lymphokine activated killer (LAK) cells, edema, as assessed by weight gain, is evident within 24 h. This is supported experimentally by evidence of increased microvascular permeability within 1 h of IL-2 infusion in the sheep lung lymph fistula model (12).

The mechanism of IL-2 induced permeability is unknown. In vitro studies with an endothelial monolayer have demonstrated no changes in permeability with IL-2 (3,6,12). Lymphocytes have been implicated as causative agents as depletion of specific lymphocyte subsets prevents the toxicity seen after several days. The observation that the lytic activity of LAK cells takes five days or more to develop suggests that other mechanisms account for early edema. Such mechanisms might involve neutrophils (PMN), which are known to be involved directly in other settings of increased permeability. Further, PMN secrete thromboxane $(Tx)A_2$ within 4 h of IL-2 treatment in vitro (13), a metabolite that is known to increase permeability across endothelial monolayers (2).

There is increasing evidence both in vitro and in vivo that the endothelial cell (EC) cytoskeleton regulates the passage of fluid and macromolecules across the microvascular junctional barrier (4,19,23,26). The membrane associated cytoskeleton consists of microfilaments of

actin, myosin and other contractile proteins, and it appears that the assembly and organization of F-actin particularly at the interendothelial junction maintains the integrity of the microvascular barrier (19). Thus agents that stimulate actin polymerization and filamentogenesis at the junctions would be expected to limit permeability, whereas conversely agents that cause microfilament disassembly and reorganization, e.g. proinflammatory agents or cytochalasin B would promote permeability and edema (7,25,26).

The aim of this study was to determine whether an agent known in vitro to reduce macromolecular flux across the microvascular junctional barrier would attenuate edema and transendothelial protein flux in a rat model of IL-2 induced permeability. We used the cyclic peptide phalloidin, and an analogue, antamanide, which are derivatives of Amanita phalloides (27). Phalloidin binds to F-actin and enhances its polymerization in vitro (5,10) and reduces macromolecular flux across EC monolayers (2,19) and in rete capillaries (20). However, phalloidin is known to be toxic in vivo, causing hepatic hemorrhage when injected IV (10,27). This toxicity is prevented by antamanide, by a mechanism thought to be due to competitive antagonism (21,28). The effect of antamanide on the cytoskeleton in vivo is not known.

METHODS

Animal Preparation

Seventy-eight adult male Sprague-Dawley rats (Charles River Lab., Wilmington MA) weighing approximately 500 g were anesthetized with intraperitoneal ketamine (35 ml/kg). A jugular venous catheter was inserted for fluid or drug infusion (1 ml/h) and hourly intravenous anesthetic dosing (ketamine, 8 mg/kg; xylazine 1 mg/kg). All animals were maintained supine for the duration of the experiment.

Preparation of Solution

Interleukin-2. Recombinant human interleukin-2 (kindly provided by Hoffman La Roche, Nutley NJ, lot #9P87) was obtained as a stock solution containing 200 x 10^6 U/ml saline, 50 mM Na acetate and mannitol (5.6 mg/ml) and diluted with saline to a final concentration of 10^6 U/ml. Depending on the batch of IL-2, endotoxin content was 3 to 6 pg/ml final dilution, assayed with the Limulus amebocyte lysate (Associates of Cape Cod, Falmouth MA). A second batch of IL-2 was used to conduct experiments for the bronchoalveolar lavage (BAL) protein estimations and lung histology. This batch, obtained as lyophilized powder (Hoffman La Roche, Nutley NJ), was reconstituted prior to use with 1 ml saline per 10^6 U IL-2 and contained vehicle of 5 mg/ml mannitol and 25 mg/ml albumin. Vehicle infused controls were used in the BAL protein experiments. In all other experiments, saline infusion was used as control.

Phalloidin. One mg of phalloidin (Sigma, St. Louis MO) was dissolved in 100 µl 100% acetone and made up with saline to a final concentration of 40 µg/ml prior to use.

Antamanide. One mg of antamanide (synthesized in one of our laboratories:D.S.) was used similarly to phalloidin except that the peptide was dissolved in absolute alcohol.

Prostanoid Assay

The concentration of TxB_2 in plasma was measured in duplicate with a double radioimmunoassay kit, using an antibody whose cross-reactivity with heterologous prostanoids was less than 1% (Seragen, Cambridge MA).

Experimental Protocol

Ten groups were studied (n=78). In the first 6 groups, saline (0.5 ml bolus every 30 min for the duration of the experiment), phalloidin (20 μg in 0.5 ml boluses every 30 min) or antamanide (20 μg in 0.5 ml boluses every 30 min) were given IV starting 30 min prior to the infusion over one hour of 10^6U IL-2 in 1 ml saline or 1 ml saline alone. Animals receiving phalloidin or antamanide were given an amount calculated to give a peak blood level of approximately 0.5 μg/ml (5×10^{-7}M) after the first injection, assuming equilibration in a blood volume of 80 ml/kg and no excretion or breakdown. Further, using these same assumptions after 1 h, animal blood concentration was approximately 10^{-6}M. This concentration of phalloidin was found sufficient to achieve cytoskeletal assembly with phalloidin in vitro (2) and to be effective in vivo (20). However, because the plasma half-life of phalloidin or antamanide is not known it was decided to give phalloidin regularly in 13 divided doses for the duration of the experiment, for a total amount of 260 μg per rat. This is approximately half the LD_{50} for phalloidin. Antamanide is not toxic in these doses, and its dose was chosen so as to give a molarity similar to phalloidin.

Six hours after the beginning of the experiment animals were killed with an overdose of

anesthetic. A thoracotomy was performed and blood for eicosanoid assay was obtained by cardiac puncture and was introduced into tubes containing 0.3 ml 0.07M ethylene diamine tetracetic acid and 0.09M aspirin, centrifuged at 1500 g x 20 min (PR-2, International Equipment Co., Needham Hts, MA) and the plasma stored at -20°C until assayed for TxB_2. The wet to dry weight (W/d) ratios of the left upper lung lobe, heart, left lobe of the liver and left kidney were calculated after weighing the freshly harvested organ, heating at 90°C in a gravity convection oven (Precision Scientific Group, Chicago IL) for 72 h and weighing the residuum.

The final four groups were used for estimations of bronchoalveolar lavage (BAL) fluid protein concentration and for lung histology. After anesthetic overdose and thoracotomy the left lung bronchus was clamped. Bronchoalveolar lavage of the right lung was performed with 3 ml saline. This was repeated 3 times. The combined lavage return of about 8 ml was centrifuged at 1500 x g for 20 min, frozen at -20°C and subsequently used for assay of protein concentration, by the spectrophotometric dye method (16).

The left lower lung lobe was used for histology. After euthanasia, the lobe was perfused with 10% formaldehyde and then inflated with the same material to a pressure of 25 cm H_2O. Following fixation, the inferior aspect of the lobe was taken, embedded and stained with hematoxylin and eosin for light microscopic analysis. All microscopic sections were interpreted in a blind fashion by a pulmonary pathologist (LK). Lung leukosequestration was quantitated by counting alveolar septal wall PMN's. Only peripheral lung parenchyma was examined. Microscopic fields containing other structures such as airways, large vessels and pleura were excluded. Leukocyte entrapment was expressed as the mean number of PMN per ten high power fields

(HPF) (x 1000).

Results are expressed as mean ± SE in text, tables and figures. Statistics were conducted by an analysis of variance and if this was positive a non-paired Student's t-test using Bonferroni's correction for multiple comparisons. Significance was accepted if $p < 0.05$.

Animals in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and the Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education and Welfare, Publication No. 78-23 (National Institute of Health), revised, 1978.

RESULTS

When saline was used as initial treatment (n=12), IL-2 led to an increase in W/d ratio: of the lungs (4.75 ± 0.14) relative to values with saline infused controls (n=12) (3.94 ± 0.20), heart (4.12 ± 0.09 vs. 3.80 ± 0.06) and kidney (4.36 ± 0.03 vs. 4.18 ± 0.05) (all $p < 0.05$, Fig. 1). There was accumulation of protein in the BAL fluid of 1770 ± 260 μg/ml (n=9), higher than 458 ± 84 μg/ml in saline controls (n=8) ($p < 0.05$, Fig. 2) and plasma TxB_2 levels increased to 1032 ± 204 pg/ml, higher than 238 ± 23 pg/ml in saline infused controls ($p < 0.05$, Table 1).

When phalloidin or antamanide was used as the initial treatment with IL-2 vehicle (both n=3) the W/d ratios for each organ were similar to saline controls (Table 2). Treatment with phalloidin (n=12) moderated the IL-2 induced increase in BAL protein leak (n=6) (955 ± 20 μg/ml, $p < 0.05$, Fig. 2) and the increase in W/d ratio of the lung (4.47 ± 0.07) and heart (3.82 ±

0.07) (both $p < 0.05$) but led to an increase in W/d ratio in the liver and kidney (both $p < 0.05$, Fig. 1).

Antamanide treatment (n=12) prevented BAL protein leak (n=6) (482 ± 13 μg/ml, $p < 0.05$). Further, it was as effective as phalloidin in attenuating the increase in W/d ratio of the lung (4.39 ± 0.12) and heart (3.87 ± 0.04). Finally, it prevented the rise in W/d ratio of the kidney (4.14 ± 0.09) (all $p < 0.05$). Treatment with either phalloidin or antamanide abolished the rise in plasma TxB_2 (both $p < 0.05$, Table 1) but did not alter lung leukosequestration of neutrophils (Fig. 3).

DISCUSSION

The data of this study suggest that the multi-system organ edema and alveolar permeability produced with a 1 h infusion of IL-2 in the rat is attenuated in part by the cyclopeptides phalloidin and antamanide. This is based on the observations that phalloidin moderated the rise in W/d ratios induced by IL-2 in the lung and heart and reduced alveolar protein leak (Fig. 1,2), and that antamanide was equally or more effective in inhibiting the W/d ratio increase and permeability. The mechanism by which these agents modify edema and permeability in vivo is not known. However, there is substantial in vitro evidence to indicate that the target for phalloidin is the EC membrane-associated cytoskeleton.

Studies of phalloidin in vitro demonstrate that it facilitates G- to F-actin polymerization, causes assembly of cytoskeletal proteins, and binds directly and avidly to F-actin (5). Phalloidin does not bind to G-actin, the unpolymerized form (10). However, while the relationship between

the binding of isolated microfilaments and the action of phalloidin on endothelial monolayers is substantiated, the mechanism by which phalloidin affects permeability either in vitro or in vivo is less clear. When injected intravenously, phalloidin is selectively taken up by hepatocytes via transport in the same multi-specific carrier system as bile acids (10,11). Phalloidin does not appear to be transported across other cell membranes. Indeed, application of rhodamine-phalloidin as an in vitro stain of F-actin requires permeabilization of endothelial cell plasma membranes. The finding that phalloidin appears to bind to hepatocyte receptors raises the possibility that the biological effect of phalloidin on microvascular permeability may also be by an endothelial receptor-mediated mechanism (11). The effectiveness of antamanide, which does not bind to F-actin, supports this hypothesis.

Phalloidin attenuated but did not prevent the permeability edema in the lung indicating either that factors other than cytoskeletal mechanisms may be involved in lung edema with IL-2 or that the drug or its dosage was not optimal. The latter two possibilities are suggested by the observation that the analogue antamanide completely prevented protein leakage. In addition, antamanide, like phalloidin, only partially inhibited the edema, indicating that the mechanisms for IL-2 to induce edema and protein leak may not be the same. In support of this, infusion of IL-2 into the isolated and perfused guinea-pig lung causes thromboxane-dependent edema without inducing permeability (9).

Phalloidin caused an increase in liver W/d ratio (Fig. 1). This is in keeping with its known hepatotoxicity (10). The problem in interpretation of this possible hepatotoxicity is that the IL-2 treated animals showed no increase in liver W/d ratio. This is in contrast with an increased W/d

ratio noted by us previously (24). In that study phalloidin had it been used would have demonstrated little increase in W/d ratio. We are unable to explain the differences between liver W/d in these two studies. The time course of monitoring was 6 1/2 hours, longer than in the present study, which may have been of importance. In addition to the increase in liver W/d ratio noted with phalloidin, there was a rise in the renal W/d ratio, a finding which is not explained by the available data.

In vitro evidence shows that IL-2, by itself, is not capable of increasing endothelial permeability (3,6,12). The present data do not define the mechanism of permeability and edema with IL-2 in this setting. The likely mediators are neutrophils and thromboxane as neutrophil depletion reduces alveolar permeability and edema in the lungs and heart in the same model, as well as preventing the rise in TxB_2 (unpublished data). The neutrophil is known to be a prominent source of Tx with IL-2 infusion (13). In the present study, the level of plasma TxB_2 was found to rise at 6 h (Table 1). Thromboxane is known to increase permeability of endothelial monolayers to macromolecules in vitro, cause loss of F-actin and lead to widening of interendothelial junctions (7,25). The observation that phalloidin and antamanide treatments were associated with reductions in levels of TxB_2 could mean that PMN oxidative activity, as estimated by TxB_2 synthesis, was modified, and might be related to the mechanism of reduction in edema following IL-2.

IL-2 infusion produced PMN sequestration in the pulmonary microvasculature after 6 h. As neutrophils are likely involved in this injury, it is theoretically possible that the observed reductions in edema and permeability could have been determined by alterations in the PMN

cytoskeleton by phalloidin or antamanide. However, we have no evidence that these agents alter PMN metabolic activity, other than their possible effect on TxB_2 synthesis by neutrophils. In vitro studies with phalloidin show no change in PMN oxidative activity when used in concentrations up to 100-fold greater than in the present study (1).

In summary, the data of this study suggest that the cyclopeptides phalloidin and antamanide reduce multi-organ injury induced by IL-2. This is consistent with in vitro data which indicate that phalloidin effects changes in permeability by modulation of the EC cytoskeleton.

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TABLE I

MODERATION OF PLASMA TxB2 *

Group	TxB2
Saline	238 ± 23
Phalloidin Control	218 ± 31
Antamanide Control	218 ± 50
Saline-IL-2	1032 ± 204†
Phalloidin-IL-2	302 ± 52**
Antamanide-IL-2	242 ± 16**

*Prostanoid levels are given in pg/ml

The symbols † and ** indicate $p < 0.05$ relative to saline control and saline-IL-2 respectively.

TABLE II

ORGAN WET TO DRY WEIGHT RATIOS IN CONTROL ANIMALS

GROUP	LUNG	HEART	LIVER	KIDNEY
Saline control	3.94 ± 0.20	3.80 ± 0.06	3.36 ± 0.06	4.18 ± 0.05
Phalloidin control	4.22 ± 0.19	3.71 ± 0.17	3.33 ± 0.03	4.20 ± 0.10
Antamanide control	4.09 ± 0.28	3.92 ± 0.05	3.36 ± 0.05	4.53 ± 0.34

Figure 1. Infusion over one hour of 10^6U recombinant human IL-2 leads to an increased W/d ratio in the lung, heart and kidney after 6 h. Pretreatment with phalloidin or antamanide attenuated edema in the lung and heart. Phalloidin caused increased W/d weight ratio in the liver and kidney. Antamanide maintained a normal W/d ratio in these organs. The symbols * and † indicate $p < 0.05$ relative to saline control and to saline-IL-2 respectively. Phalloidin control and antamanide control gave values similar to saline control (Table 2).

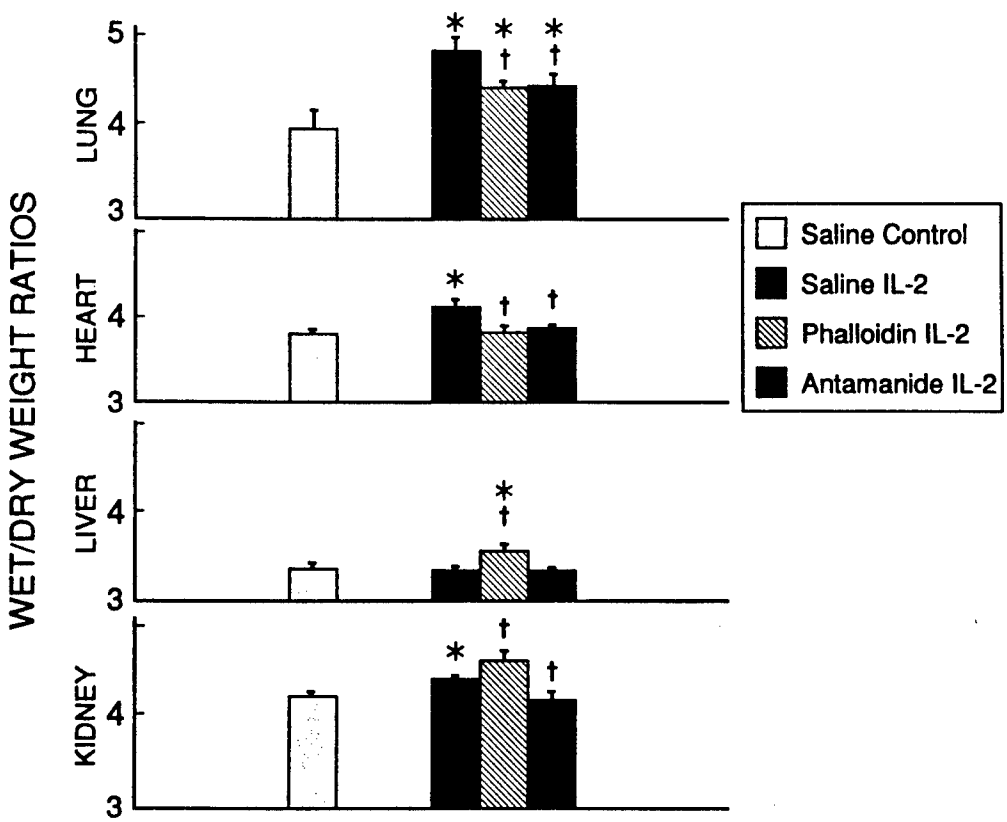

Figure 2. Infusion of 10^6U IL-2 produced protein leak into alveoli after 6 h. Endothelial barrier enhancement with phalloidin or antamanide reduced the leak. The symbols * and † indicate p <0.05 relative to saline control and saline-IL-2 respectively.

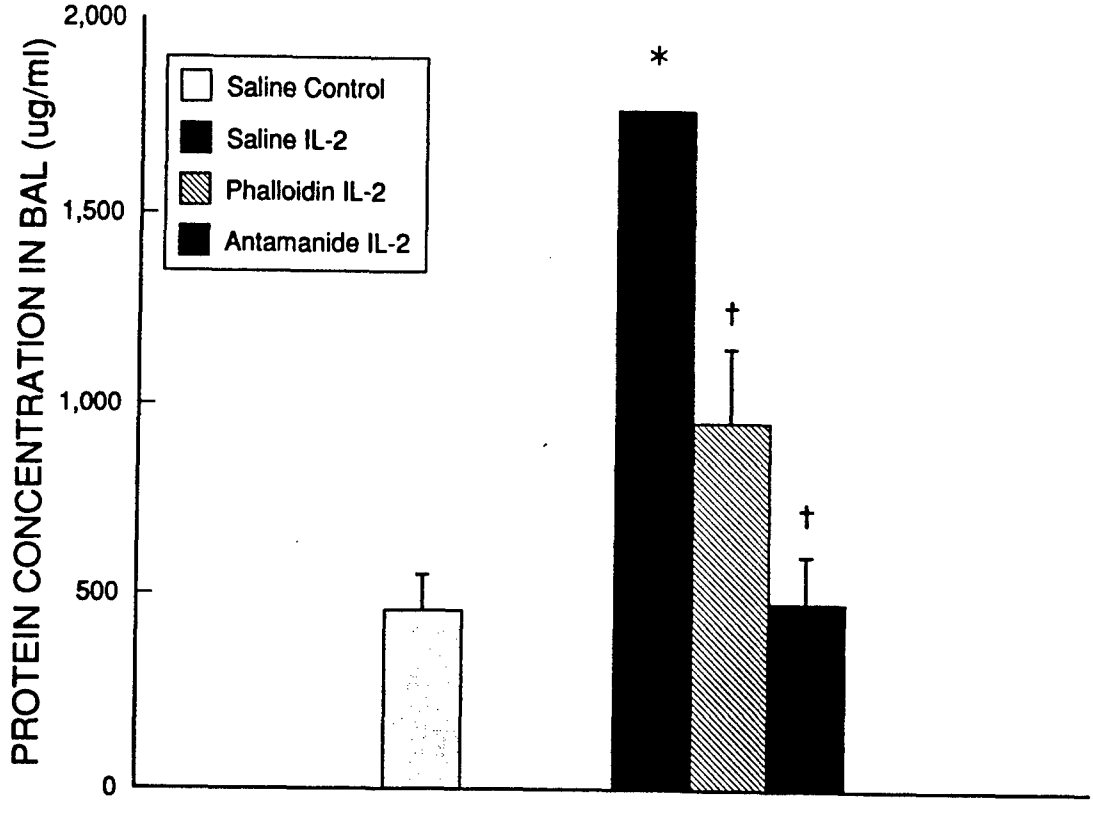

Figure 3. IL-2 produces lung neutrophil sequestration after 6 h which was not significantly affected by phalloidin or antamanide. The symbol * indicates $p < 0.05$ relative to saline control.